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Primate Cytomegaloviruses Encode and Express an IL-10-like Protein

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An open reading frame (ORF) with homology to interleukin-10 (IL-10) has been identified in rhesus cytomegalovirus (RhCMV). The IL-10-like protein is generated from a multispliced, polyadenylated early gene transcript encompassing part of the corresponding UL111A ORF of human CMV (HCMV). Immunological analyses confirm expression of the IL-10-like protein both in tissue culture and in RhCMV-infected rhesus macaques. Conserved ORFs were subsequently identified in human, baboon, and African green monkey CMV, and a fully processed transcript has been mapped in fibroblasts infected with the Towne strain of HCMV. The conservation of this previously unrecognized ORF suggests that the protein may play an essential role in primate CMV persistence and pathogenesis.

INTRODUCTION

Cytomegaloviruses (CMV) are exceedingly adept at persisting in healthy hosts, despite vigorous antiviral immune responses. While antiviral immunity can contain primary CMV infection and limit disease in most cases (Alford and Britt, 1993), it is not sufficient to eliminate viral reservoirs from the infected individual (Kondo et al., 1996; Söderberg-Nauclér *et al.,* 1997). Latent viral genomes periodically reactivate to produce infectious virus during the life of an infected host, usually without clinical signs of disease (Pass *et al.,* 1982; McVoy and Adler, 1989). A fundamental issue for understanding mechanisms of viral persistence is the identification and *in vivo* characterization of CMV gene products that are involved in the establishment and maintenance of stable virus–host relationships.

HCMV expresses multiple proteins that can potentially attenuate or subvert host immune responses *in vivo*. These include the US2, 3, 6, and 11 gene products that are involved in downregulation of major histocompatibility complex type 1 (MHC I) expression in infected cells, facilitating evasion from cytotoxic T-cell immune responses (reviewed in Wiertz *et al.,* 1997). The UL18 ORF encodes an MHC I-like protein that may confer *in vitro* resistance to natural killer (NK) cell lysis in cells expressing low amounts of surface MHC I (Reyburn *et al.,* 1997). HCMV encodes both an α-chemokine (UL146) that likely functions to modulate host neutrophil responses (Penfold *et al.,* 1999), and a β-chemokine receptor (US28) that binds and sequesters chemokines such as RANTES (Neote *et al.,* 1993; Gao and Murphy, 1994; Bodaghi *et al.,* 1998). The UL144 ORF expresses a protein that appears to be a member of the tumor necrosis factor receptor superfamily (Benedict *et al.,* 1999), and the UL37 protein functions as an inhibitor of apoptosis (Goldmacher *et al.,* 1999). Some of these genes (US2–11) have been demonstrated to be dispensable for propagation *in vitro* (Kollert-Jons *et al.,* 1991; Greaves *et al.,* 1995). However, it is not known what roles these proteins play during virological expression of HCMV persistence and pathogenesis *in vivo*.

We recently reported on the development of a nonhuman primate model of CMV that has been used to study viral and host determinants of persistence and pathogenesis (Tarantal *et al.,* 1998; Lockridge *et al.,* 1999). We have used this model to identify a novel ORF in RhCMV with sequence homology to cellular IL-10 (cIL-10). cIL-10, also referred to as cytokine synthesis inhibitory factor, is a complex and multifunctional cytokine that downregulates inflammatory responses and modulates MHC I and II surface expression in multiple immune cell types (reviewed in Moore *et al.,* 1993). Based on the strong colinearity of the primate CMV genomes, we identified the cognate, previously unrecognized ORF in HCMV and in baboon and African green monkey CMV (BaCMV, AGMCMV, respectively). This is the first report of primate CMV encoding and transcribing an IL-10-like gene product. The identification of this previously unrecognized gene has important ramifications for resolving mechanisms of CMV immune evasion.

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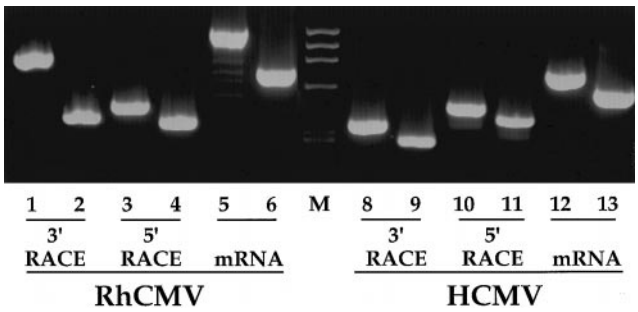

FIG. 1. PCR analysis of RhCMV and HCMV IL-10-like genes. RNA from RhCMV- or HCMV-infected fibroblasts was used to synthesize cDNA, either the 5′ or 3′ end of the transcript (5′ and 3′ RACE, respectively; lanes 2 and 4 for RhCMV, 9 and 11 for HCMV) or full-length mRNA (RhCMV: lane 6; HCMV: lane 13). PCR amplification of the cDNA is presented in comparison to amplification of genomic DNA (RhCMV: lanes 1, 3, and 5; HCMV: lanes 8, 10, and 12). In all cases, cDNA sizes are less than corresponding amplification of genomic DNA. M: ϕx174/*Hae*III DNA marker.

RESULTS

Isolation of RhCMV IL-10 sequence

Random restriction endonuclease fragments of RhCMV were cloned, sequenced at the termini, translated, and aligned with predicted ORF of HCMV (Chee *et al.,* 1990). Analysis of a partial library of the RhCMV genome demonstrated that RhCMV is essentially colinear with HCMV (data not shown). One clone was isolated with a 5′ end that aligned with HCMV UL105 (helicase; accession number AF079496), but with a 3′ terminus that exhibited no apparent homology to any identified HCMV ORF. A BLAST search of protein sequences in GenBank with the predicted translation products of the 3′ end of the RhCMV clone revealed significant homology to mammalian cIL-10 sequences (not shown). The 3′ terminus of the clone was predicted to be near the short, uncharacterized UL110/111 ORF of HCMV, based on the size of the cloned RhCMV fragment and the alignment of the 5′ end within UL105.

RNA analysis of RhCMV IL-10 expression

5′ and 3′ RACE reactions were performed to determine whether this region of the RhCMV genome was transcribed. Amplification products from the RACE reactions were cloned, sequenced, and compared to the sequence of the genomic clone. Sequences of partial transcripts revealed that the 5′ and 3′ ends of the transcript were generated by multiple splice events and that the transcript was polyadenylated. PCR products of cDNA, using primers determined from the termini of transcripts and primers used for the RACE reactions, were shorter than the amplicons obtained using genomic DNA as a template (Fig. 1, lanes 1–4). Similarly, the full-length transcript was also significantly shorter than the corresponding unspliced genomic sequence (Fig. 1, lanes 5 and 6). The 746-nucleotide (nt) transcript (accession number AF200417) consisted of four exons, 275, 222, 62, and 187 nt in length. The three introns (95, 427, and 84 nt) all contained consensus splice-site sequences (5′ GT···AG 3′), and the site of polyadenylation was 17 bases downstream from a consensus polyadenylation signal (AATAAA).

Isolation of HCMV IL-10 cDNA

A more detailed examination of UL110/111 region of HCMV was undertaken, since the RhCMV clone would have represented the first example of noncolinearity between the RhCMV and HCMV genomes. A series of primers (Table 1) was designed to amplify the 5′ and 3′ ends of potential transcripts using RNA obtained from MRC-5 cells infected with the Towne strain of HCMV. Two primers, located downstream of the UL111A ORF, detected a multiply spliced, polyadenylated transcript. The amplicons of both 5′ and 3′ RACE cDNAs were shorter in length than the corresponding amplification of genomic HCMV DNA (Fig. 1, lanes 8–11). The amplification product of the full-length transcript (accession number AF202536), based on the mRNA start and polyadenylation sites identified by the RACE reactions, was also shorter in length than the corresponding genomic DNA amplicon (Fig. 1, lanes 12 and 13). A fully processed message (633 nt) initiated at position 159,642 and terminated at position 160,430, 14 bases downstream from a consensus polyadenylation sequence (AD169 coordinates, X17403). The transcript was generated from three exons/two introns, versus four exons/three introns in RhCMV. The cDNA for RhCMV and HCMV IL-10 exhibited considerable sequence divergence with only 45% sequence identity (not shown).

Sequence comparisons of predicted open reading frames

Both the RhCMV and HCMV transcripts encoded extended translation products beginning at methionine codons located 77 and 38 bases, respectively, downstream of the transcription start sites. The RhCMV ORF was 189 amino acids (aa), and the HCMV ORF was 176 aa. The amino-terminal portion of the HCMV IL-10-like ORF corresponded to the UL111A ORF; splicing of the transcript maintained an open reading frame prior to the identified stop codon of UL111A. Both proteins appeared to include signal peptides (Nielsen *et al.,* 1997) that would yield cleaved peptides of 158 and 151 aa, respectively. It is not known whether the CMV IL-10 proteins are secreted, although cIL-10 is a secreted protein (MacNeil *et al.,* 1990). The lengths of the cleaved cellular IL-10 in humans and rhesus macaques (accession numbers P22301 and P51496, respectively) are both 160 aa (178 aa uncleaved). Pairwise sequence comparisons of the viral IL-10 proteins and their apparent cellular homologs indi-

TABLE 1

Primers Used for PCR, RACE, and RT Reactions

Primer	Sequence (5′ → 3′)	Position[a]
295	ATGACAGACTGTGACTCCTC	RhCMV: 1,426–1,406
307	CGTGACCAATGAACTCATGG	RhCMV: 1,539–1,558
310	ATGGAGCACGTCATTG	RhCMV: 1,513–1,495
312	GTGGTTAAACAGTACGTTTATTA**G**AG[b]	RhCMV: 2,441–2,415 HCMV: 160,432–160,407
316	GCAGTAGATACCAGATTCT	RhCMV: 727–745 HCMV: 159,225–159,243
328	ATCCGAGAATT**C**TAAACCGGAG[b]	RhCMV: 2,398–2,377
329	ATGGCGGTGGTCGTGCTTTCAG	RhCMV: 1,230–1,251
344	GAACTGCAGCAGGAACG[b]	RhCMV: 2338–2322
376	TAGCCTGGAGAAGGA*T*CCGAGAACGAC[b]	RhCMV: 1125–1151
378	GGACTCAAGACGGAATTGCATAGTATGC	HCMV: 160,066–160,093
381	TTGTAGATGGATTCTAGCGTCGAGC	HCMV: 160,118–160,094
386	ATGCTGCGGCGATGCTGTCGGTGATGGTCTCTT	HCMV: 159,667–159,699
393	ATTCTCTGTTGCAGCGGCGGT	RhCMV: 1,091–1,111

[a] The Accession Nos. for the RhCMV and HCMV sequences are AF200417 and X17403, respectively.
[b] Underlined and bold nucleotides are explained in the text.

cated that the viral ORF were nearly as divergent from each other as they were from their hosts. Mammalian IL-10 proteins are strongly conserved; the human and macaque proteins are 95% identical (ID) and 96% similar (SI) (Table 2; protein comparisons are for cleaved proteins only). Both the RhCMV and HCMV proteins were 25% ID/41% SI with their respective host cIL-10. The two viral ORF were 31% ID/38% SI. There was almost no sequence variation between different isolates of either RhCMV or HCMV. There was only one amino acid difference between RhCMV strain 68-1 and a primary isolate (accession number AF200740); the Towne strain of HCMV and the predicted protein of AD169 were identical, except for one additional amino acid in the Towne sequence.

Characterization of IL-10 in AGMCMV and BaCMV

To determine whether other primate CMV encoded a similar ORF, primers 316/312 (Table 1) were used to amplify the corresponding regions of the BaCMV and AGMCMV genomes (accession numbers AF202535 and AF202534, respectively). Using the protein sequence of the RhCMV and HCMV proteins, putative cleaved proteins of 159 aa each were identified in both species of African nonhuman primate CMV. The proteins would be generated from a primary transcript consisting of four exons/three introns. For both transcripts, introns and polyadenylation would include consensus sequences. Alignment of these two CMV with RhCMV and HCMV IL-10-like ORF demonstrated strong sequence conservation among the simian CMV. The RhCMV, BaCMV, and AGMCMV were 67–79% ID/72–82% SI compared to each other. Both BaCMV and AGMCMV IL-10-like ORF were comparably divergent from cIL-10 and the HCMV ORF, similar to RhCMV. Other herpesviruses have transduced cIL-10 sequences, including Epstein–Barr virus (EBV) (Hsu *et al.,* 1990). The EBV IL-10 is strongly conserved with cIL-10 sequences, although EBV IL-10 does exhibit

TABLE 2

Percentage Identity/Similarity of Cellular and Viral IL-10 Proteins

	Mmu cIL-10[a]	RhCMV	HCMV	AGMCMV	BaCMV	EBV′ [b]	Length (aa)[c]
Human cIL-10[d]	95/96	25/41	27/37	28/39	27/37	90/92	160
Mmu cIL-10		25/41	27/37	28/40	27/39	86/90	160
RhCMV			31/38	73/77	67/72	25/40	158
HCMV				33/40	32/41	29/41	151
AGMCMV					79/82	31/42	159
BaCMV						30/41	159
EBV							147

[a] Accession No. P51496.
[b] Accession No. P03180.
[c] Mature protein.
[d] Accession No. P22301.

functional differences from the cIL-10 protein (Liu *et al.,* 1997).

Search for IL-10 in other β-herpesviruses

Sequence alignments of the cIL-10 with the CMV IL-10-like ORF revealed there were 20 amino acids conserved in all isolates, including four cysteine residues (the signal peptide was not included) (Fig. 2A). These amino acids were also conserved in EBV IL-10 (not shown). There were an additional 15 amino acids conserved in all four CMV representatives. Despite the sequence divergence of the CMV IL-10-like proteins from cIL-10, hydropathic profiles of the proteins were similar (Fig. 2B). These data imply conservation of structure. Computer searches were performed on the three translation reading frames between UL105 and 112 of murine CMV (MCMV) and human herpesvirus 6 (HHV6), using conserved amino acid motifs in the cellular and viral IL-10 proteins. No homologous proteins were identified, although it should be noted that these other β-herpesviruses have regions between UL105 and 112 without identified ORF. Based on splicing of the IL-10-like ORF of primate CMV, detailed RNA analysis is warranted for this area of the genome of MMV and HHV6, to rule out the existence of homologous proteins.

Intron–exon structure

The protein alignments, together with mapping of the transcripts, are informative about the evolutionary relatedness of these ORF. Two exon–exon splice junctions have been conserved in the cellular and CMV IL-10 (Fig. 2A). These correspond to the junctions between exons 1 and 2, and between exons 3 and 4 of cIL-10. The protein alignments suggest that primate CMV have lost the intron between exons 2 and 3 of cIL-10, resulting in an in-frame fusion of these exons (Fig. 2C). The HCMV ORF has also lost the intron between exons 4 and 5 of cIL-10, resulting in a gene composed of three exons/two introns. These data are consistent with transduction of a cIL-10 gene by a progenitor CMV with sequence variation and progressive loss of introns during speciation.

Expression kinetics

The expression kinetics of the RhCMV IL-10-like gene were determined by Northern blot analysis following infection of primary rhesus skin fibroblasts (Fig. 3). An antisense RNA probe to exons 3 and 4 of the RhCMV ORF hybridized with an approximately 0.9-kilobase (kb) transcript beginning at 12 h postinfection. The transcript was not detected in the presence of the protein synthesis inhibitor cycloheximide but was detected in the presence of the replication inhibitor phosphonoformic acid (PFA). Since the concentration of PFA used for the infection had been previously demonstrated to inhibit RhCMV replication (data not shown), the expression of the 0.9-kb IL-10 transcript was consistent with that of an early gene. The antisense IL-10 probe also detected an uncharacterized transcript of approximately 2.6 kb. The absence of expression of the uncharacterized 2.6-kb transcript in the presence of PFA indicated that this was a late transcript. Western blot analysis of timed RhCMV-infected cell lysates probed with polyclonal rabbit antisera confirmed the early gene kinetics of the RhCMV IL-10-like protein (data not shown). RT/PCR analysis of RNA from cells infected with HCMV demonstrated the presence of the IL-10 transcript by 12 h postinfection (data not shown). Northern blot analysis was not performed for HCMV.

Immunogenicity and expression of IL-10 in RhCMV-infected macaques

We next determined whether sera from a rhesus macaque, chronically infected with RhCMV, would recognize the viral IL-10-like protein. Cos-7 cells were transfected with a *myc*-epitope-tagged expression vector pcDNA m/H IL-10 containing the genomic sequence of the RhCMV IL-10-like gene. Figure 4A (lanes 1–5) shows an immunoblot of the transfected Cos-7 cell lysate probed with either polyclonal rabbit antisera or sera from a chronically RhCMV-infected rhesus macaque. Both the rabbit and monkey postimmune sera recognized the same 28-kDa IL-10 fusion protein recognized by the anti-*myc* antibody. This protein was not detected with preimmune sera. These results demonstrated IL-10-like protein expression during RhCMV infection. We subsequently examined the kinetics of the humoral immune response against the IL-10-like protein during RhCMV infection. Figure 4A (lanes 6–9) also shows Western blot analyses of RhCMV-infected cell lysates probed with sequential plasma from a rhesus macaque infected with RhCMV (Lockridge *et al.,* 1999). Beginning at 2 weeks postinoculation, an IgG antibody response against an approximately 25-kDa protein was detected; this response continued with increasing reactivity at 6 months postinoculation. Preimmune plasma did not exhibit reactivity to this protein. Rabbit polyclonal anti-IL-10 sera recognized a protein of identical size (data not shown). These results indicate that the IL-10-like protein was expressed during RhCMV infection and was recognized by the host immune response. Further evidence of *in vivo* expression was shown by immunohistochemical analysis of thymic tissue from a rhesus macaque coinfected with RhCMV and simian immunodeficiency virus (Fig. 4B). Cells with intranuclear and cytoplasmic inclusions were stained with a rabbit polyclonal antisera generated against bacterially synthesized RhCMV IL-10-like protein; staining was confined to the cytoplasm.

DISCUSSION

This report describes the first characterization of an IL-10-like gene within β-herpesviruses and highlights the

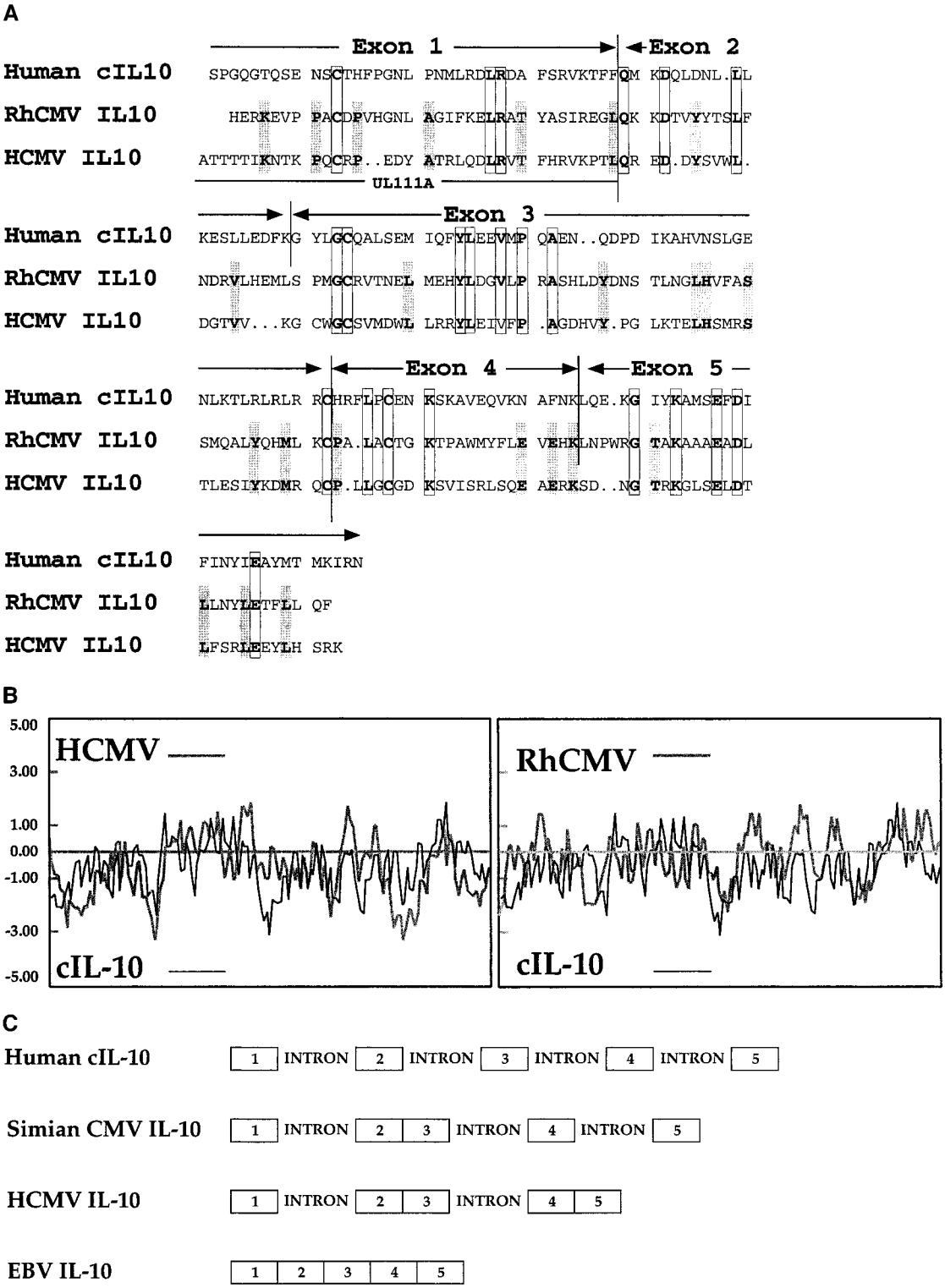

FIG. 2. Protein alignments of the primate CMV IL-10-like proteins with cIL-10. (A) Amino acid comparison of human cIL-10 and HCMV and RhCMV vIL-10-like protein. Identical amino acids in all three proteins are boxed, or in the CMV IL-10 are shaded. The five exons of the cIL-10 are indicated. The exon–exon junctions in each protein are noted by a vertical line. The region of overlap of HCMV IL-10-like protein with UL111A is noted. (B) Hydrophobicity plot comparisons of cellular and viral IL-10 proteins for human (left) or rhesus macaque (right). (C) Splicing patterns of cellular and viral IL-10 genes. Exons are depicted as boxes.

utility of the rhesus macaque model of CMV to facilitate new knowledge of HCMV. A fortuitous subclone of the RhCMV genome with homology to cIL-10 spurred the reexamination of the corresponding region of HCMV to detect a previously unrecognized gene within HCMV. The Il-10-like ORF adds to the growing list of identified

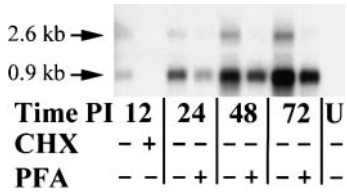

FIG. 3. Northern blot analysis of RhCMV-infected primary rhesus macaque skin fibroblasts. Primary rhesus skin fibroblasts were infected with RhCMV and RNA was harvested at different times postinfection (Time PI). Cells were infected in the presence (+) or absence (−) of either cycloheximide (CHX) or phosphonoformic acid (PFA). RNA was analyzed by Northern blot, using an antisense RNA probe to exons 3 and 4 of RhCMV IL-10. The approximate 0.9-kb IL-10 gene and an unidentified 2.6-kb transcript hybridizing to the probe are indicated.

ORF within HCMV with the potential to modulate host antiviral immune responses (see Introduction).

IL-10 homologs have been reported for other herpesviruses, including Epstein–Barr virus (EBV) (Hsu *et al.,* 1990; Moore *et al.,* 1990) and equine herpesvirus 2 (Rode *et al.,* 1993; Fleming *et al.,* 1997). The EBV IL-10 homolog is highly conserved in sequence with its cellular counterpart (Vieira *et al.,* 1991) (Table 2), yet has evolved biological functions distinct from cIL-10 (discussed later). Gesser *et al.* (1997) found that biological functions of cIL-10 are confined to small, defined segments of the protein. Functional domains for human cIL-10 have been localized to a 9-aa sequence at the carboxyl end and an 8-aa sequence near the amino terminus of the processed protein. The amino proximal motif that exhibits regulation of MHC II antigen expression and induces proliferation of murine mast cells has only two conserved amino acids with corresponding sequence in EBV IL-10. The carboxyl-terminal motif, which determines the cytokine synthesis inhibitory activity and affects cellular migration, is only partially conserved in EBV (6 of 9 amino acids). There is minimal sequence homology in these motifs between cIL-10 and CMV IL-10. Given the divergence of the EBV IL-10 from cIL-10 in these two functional regions, divergence of primate CMV IL-10 from cIL-10 was not unexpected.

The function of the IL-10-like ORF is under investigation. Cellular IL-10 is a multifunctional cytokine that has suppressive effects on inflammation and cytokine production (Fiorentino *et al.,* 1989). EBV IL-10 inhibits synthesis of cytokines by activated T cells, inhibits antigen-specific T-cell proliferation and induces proliferation and Ig secretion in activated B cells (Rousset *et al.,* 1992; de Waal Malefyt *et al.,* 1997). It is well documented that infection with CMV can have immunosuppressive effects *in vitro* (Einhorn and Ost, 1984; Schrier and Oldstone, 1986; Schrier *et al.,* 1986). Acquisition of an IL-10-like gene may allow CMV to subvert the antiviral cell-mediated immune response long enough to establish a latent infection. Our data indicate that the RhCMV IL-10-like gene is produced during infection of rhesus macaques and is a target of the humoral immune response.

Identification of this gene in primate CMV suggests that acquisition of cIL-10 function may be important for the establishment and maintenance of viral persistence. Indeed, phenotypic data of CMV infection are compatible with possible functions of a viral IL-10-like protein, based on known functions of cIL-10. cIL-10 has been shown to decrease antigen-specific $CD4^+$ T-cell proliferation by reducing the expression of MHC II molecules on antigen-presenting cells (de Waal Malefyt *et al.,* 1997). Acquisition of this cIL-10 function may allow viral escape from cell-mediated immune surveillance, since $CD4^+$ T cells are required for $CD8^+$ T-cell responses. Such a mechanism may play a role in the early stages of CMV infection prior to the development of host antiviral immune responses that protect the infected host from disease (Lockridge *et al.,* 1999). According to this scenario, any virally induced attenuation of inflammatory responses

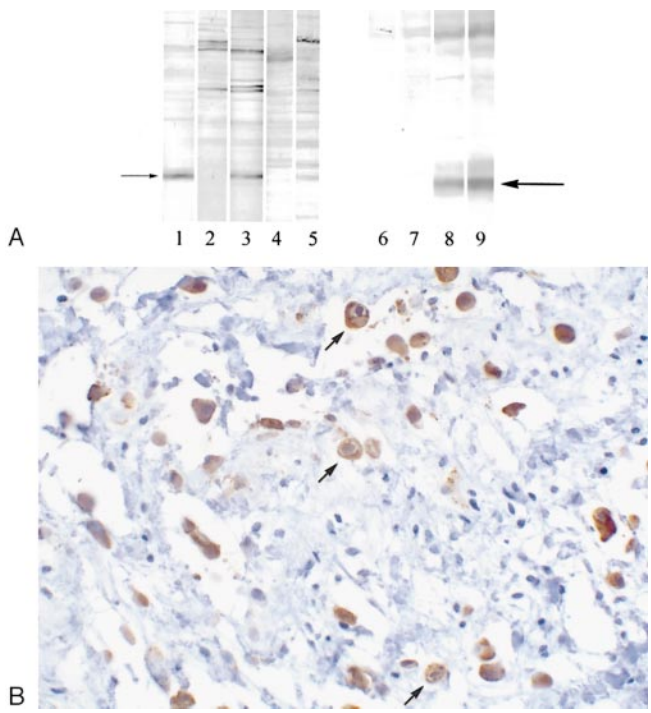

FIG. 4. Protein analysis of RhCMV-infected cells. (A) Western blot analysis of Cos 7 (lanes 1–5) or primary rhesus macaque skin fibroblast cell lysates (lanes 6–9). Cos 7 cells were transfected with pcDNA m/H IL-10 and probed with anti-*myc* antibody (lane 1), pre- and postimmune polyclonal rabbit sera (diluted 1:50; lanes 2 and 3), and pre- and postimmune RhCMV-infected rhesus macaque plasma (diluted 1:100; lanes 4 and 5). RhCMV infected skin fibroblasts were probed with longitudinal plasma from a RhCMV-infected rhesus macaque (diluted 1:50), preinoculation (lane 6) or 2 weeks (lane 7), 3 months (lane 8), and 6 months (lane 9) postinoculation. (B) Immunohistochemical analysis of RhCMV vIL-10-expressing cells. Thymic tissue from a rhesus macaque coinfected with RhCMV and SIV. vIL-10-positive cells are brown (DAB) and cells are counterstained with hematoxylin (blue). Examples of positively stained cells exhibiting RhCMV cytopathic effect are noted by arrows.

would provide the virus with a slight replicative advantage to disseminate from the primary site of infection to sites of persistence and latency.

Recently, HCMV was shown to inhibit interferon-γ (IFN-γ)-induced MHC II expression on infected cells by both disruption of the JAK/STAT signaling pathway (Miller *et al.,* 1998) and repression of class II transactivator (CIITA) mRNA expression (LeRoy *et al.,* 1999). Interestingly, cIL-10 has been shown to inhibit expression of IFN-γ-induced tyrosine phosphorylation of STAT1 (Ito *et al.,* 1999) and IFN-γ-induced expression of CIITA mRNA (O'Keefe *et al.,* 1999), thus supporting the presence of an HCMV protein with IL-10-like functionality. Additional studies regarding the functional properties of the CMV IL-10-like protein are ongoing in our laboratory and will be necessary to determine the role of the IL-10-like protein in the immunopathogenesis of CMV infection.

MATERIALS AND METHODS

Virus strains, constructs, and cells. The 68-1 strain of RhCMV (Asher *et al.,* 1974) (American Type Culture Collection [ATCC], Manassas, VA) and Towne strain of HCMV were used for these studies (generously provided by R. Spaete). Primary rhesus skin fibroblasts were used for RhCMV infections (Kaur *et al.,* 1996). HCMV was propagated on MRC-5 human diploid fibroblasts (ATCC). Cos-7 cells were transfected with a *myc*/His epitope-tagged expression vector (pcDNA3.1 *myc*/His; Invitrogen, Carlsbad, CA) containing the genomic sequence of the RhCMV IL-10-like gene (pcDNA m/H IL-10). Cells were transfected with DOTAP/DOPE (Avanti Polar-Lipids, Inc., Alabaster, AL). The genomic sequence was amplified with primers 376 and 344 and cloned into pcDNA3.1*myc*/His, using *Bam*HI and *Pst*I restriction endonuclease sites within 376 and 344, respectively (Table 1; restriction sites are underlined). This construction inserted the RhCMV IL-10 genomic sequence, beginning 30 nucleotides upstream of the translation start site and extending to a point four nucleotides upstream of the translation stop codon, in-frame with the 3′ epitope tags. The *Bam*HI restriction site within 376 was generated by modifying two nucleotides within the 5′ untranslated portion of the sequence (GA → TC) (Table 1; modified bases are in bold). In addition, the use of the *Pst*I restriction site within 344 eliminated the carboxyl-terminal amino acid (phenylalanine) from the fusion protein.

PCR and RACE reactions. Conditions for RNA isolation, PCR, reverse-transcription, and 5′/3′ RACE reactions have been described previously (Barry *et al.,* 1996). The following primers were used for PCR, RACE, and reverse-transcription reactions (see Table 1 for primer sequences). Primers 316 and 312 were used to amplify genomic fragments spanning the IL-10 region from HCMV (Towne), AGMCMV, BaCMV, and a primary isolate of RhCMV. It should be noted that there is one base pair difference between the sequence of 312 and HCMV; the "G" residue, three nucleotides from the 3′ end of PAB312 (Table 1, bold), corresponds to "A" in HCMV. Primers 307 and 378 were used for 3′ RACE reactions for RhCMV and HCMV, respectively; RT reactions were primed with the 3′ adaptor primers of Frohman *et al.* (1988). Primers 310 and 312 were used to prime the RT reaction for 5′ RACE for RhCMV and HCMV, respectively. The RhCMV 5′ RACE cDNA were amplified using primer 295 and the 5′ adaptor primer of Frohman *et al.* (1988). The HCMV 5′ cDNA was amplified using primer 381 and the SMART RACE cDNA amplification kit from Clonetech (Palo Alto, CA). Full-length cDNA for RhCMV and HCMV were generated using primer pairs 393/312 and 386/312, respectively. PCR amplification products were cloned into the TOPO-TA vector (Invitrogen) for sequencing.

Sequence analysis. Sequencing was performed by Davis Sequencing (Davis, CA). Sequence comparisons and hydrophobicity plots were performed with the SEQWeb sequence analysis software (Genetics Computer Group, Madison, WI). BLAST sequence searches were performed using the resources at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Polyclonal rabbit antisera. The RhCMV IL-10 cDNA was amplified with primers 329 and 328 and cloned into the pGEX2T vector (Pharmacia Biotech, Piscataway, NJ). Primer 329 also included a *Bam*HI restriction site at the 5′ end of the primer and 328 was modified by one base to incorporate an *Eco*RI restriction site to facilitate cloning into the expression vector (Table 1, G → C, italicized). This construction placed the IL-10 gene in-frame with the GST fusion protein beginning 23 amino acids downstream of the IL-10 translation start codon. IL-10/GST fusion protein was generated and purified according to the manufacturer's directions. Antisera were generated by immunization of rabbits with RhCMV IL-10/GST fusion protein at Animal Resource Services, University of California, Davis.

RNA and protein analysis. Northern analysis was performed according to published protocols (Kravitz *et al.,* 1997). Rhesus skin fibroblasts were infected with RhCMV strain 68-1 at an m.o.i. of 1. RNA was purified at 12, 24, 48, and 72 h postinfection and analyzed by Northern blot, using an antisense RNA probe corresponding to exons 3 and 4. Infections were performed in the presence and absence of cycloheximide (200 μg/ml, 12 h only) or phosphonoformic acid (400 μg/ml, 24, 48, 72 h). Immunoblotting and immunohistochemistry were performed as previously described (Lockridge *et al.,* 1999).

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